DIFFERENTIAL REGULATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) BY CYTOKINES IN MURINE MACROPHAGE J774.2 CELL LINE: ELUCIDATION OF SIGNAL TRANSDUCTION PATHWAYS OF TUMOUR NECROSIS FACTOR ALPHA (TNFα) IN REGULATING MACROPHAGE PPARγ GENE EXPRESSION

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UNIVERSITI SAINS MALAYSIA

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DIFFERENTIAL REGULATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) BY CYTOKINES IN MURINE MACROPHAGE J774.2 CELL LINE: ELUCIDATION OF SIGNAL TRANSDUCTION PATHWAYS OF TUMOUR NECROSIS FACTOR ALPHA (TNFα) IN REGULATING MACROPHAGE PPARγ GENE EXPRESSION

by

LIM CHUI HUN

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

May 2007
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Lim Chui Hun

May 2007
This thesis is dedicated to

my father, forever in loving memories;

my husband, Kelvin Cheah

&

my son, Benjamin.

Thank you for being my source of inspiration.
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LIST OF ABBREVIATIONS

12-HETE  12-hydroxyeicosatetraenoic acid
13-HODE  13-hydroxyoctadecadienoic acid
15-dPGJ2  15-deoxy-\( \Delta ^{12,14} \)-prostaglandin J\(_2\)
15-HETE  15-hydroxyeicosatetraenoic acid
5'UTR    5' untranslated region
9-HODE   9-hydroxyoctadecadienoic acid
AF-1     Activation function 1
AF-2     Activation function 2
AP1      Activating protein 1
apoE     Apolipoprotein E
APS      Ammonium persulphate
ARF6     Adipocyte differentiation-dependent regulatory factor
ATCC     American Type Culture Collection
BADGE    Bisphenol diglycidyl ether
BCP      1-Bromo-3-Chloropropane
bp       Base pair
BSA      Bovine serum albumin
C/EBP    CCAAT/enhancer binding protein
CaCl\(_2\) Calcium Chloride
CDDO     Triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid
cDNA     Complementary DNA
CO\(_2\)   Carbon dioxide
CoA      Coactivators
CoR      Corepressor
COX      Cyclooxygenase
CRE      Cyclic AMP response element
CREB     cAMP-response element binding protein
dATP     Deoxyadenosine triphosphate
DBD      DNA-binding domain
dCTP     Deoxycytidine triphosphate
dGTP     Deoxyguanosine triphosphate
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  Dimethyl sulphoxide
DNA  Deoxyribonucleic acid
dNTPs  Deoxyribonucleoside triphosphates
DR-1  Direct repeat-1 base spacer
DTT  Dithiothreitol
dTTP  Deoxynthymidine triphosphate
EC  Endothelial cell
EDTA  Ethylene diaminetetraacetic acid
EMSA  Electrophoretic mobility shift assay
EPA  Eicosapentaenoic acid
ET-1  Endothelin-1
FBS  Fetal bovine serum
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
H₂O  Water
HAT  Histone acetyltransferases
HDAC  Histone deacetylase
HDL  High-density lipoprotein
HODE  Hydroxyoctadecadienoic acids
HRP  Horseradish Peroxidase
ICAM-1  Intracellular adhesion molecule-1
IFNγ  Interferon gamma
IL-1α  Interleukin 1α
IL-1β  Interleukin 1β
IL-2  Interleukin 2
IL-4  Interleukin 4
IL-6  Interleukin 6
INOS  Inducible nitric oxide synthase
IP-10  IFNγ-inducible protein of 10 kDa
IPTG  Isopropyl-β-D-thiogalactopyranoside
ISGF-RE  Interferon stimulated gene factor response element
I-TAC  IFN-inducible T-cell a-chemoattractant
kb  kilobase pairs
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte-chemoattractant protein-1</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>Mig</td>
<td>Monokine induced by IFNγ</td>
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<tr>
<td>M-MLV RT</td>
<td>Molony murine leukemia virus reverse transcriptase</td>
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<tr>
<td>MMP-9</td>
<td>Metalloproteinase</td>
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<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propanesulphonic acid</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
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<td>OxLDL</td>
<td>Oxidized low density lipoprotein</td>
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<tr>
<td>PBP</td>
<td>PPAR binding protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
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<td>PPAR gamma coactivator-1</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>poly(dI-dC)</td>
<td>Polydeoxyinosinic-deoxycytidylic acid</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activated-Receptor</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferators activated receptor gamma</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome proliferator response element</td>
</tr>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid and thyroid hormone receptors</td>
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<tr>
<td>SR-A</td>
<td>Scavenger receptor A</td>
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<tr>
<td>SRC-1</td>
<td>Steroid receptor coactivator 1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<td>TAE</td>
<td>Tris-acetate-EDTA</td>
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<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
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<td>TEMED</td>
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<td>TNFα</td>
<td>Tumour necrosis factor α</td>
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<tr>
<td>TRE</td>
<td>TPA-response element</td>
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<td>TZDs</td>
<td>Thiazolidinediones</td>
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<td>UV</td>
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<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
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PENGAWALATURAN PEMBEZAAN RESEPTOR AKTIVASI PEMBIAKAN PEROKSISOM GAMMA (PPARγ) OLEH SITOKINA DI DALAM SEL TURUNAN MAKROFAJ MURIN J774.2: PENGENALPASTIAN LALUAN ISYARAT TRANSDUKSI FAKTOR NEKROSIS TUMOR ALFA (TNFα) DI DALAM PENGAWALATURAN PENGEKSPRESAN GEN PPARγ

ABSTRAK

Aterosklerosis merupakan punca kematian utama di negara-negara maju. Peranan PPARγ dalam makrofaj yang diaktifkan oleh sitokina adalah penting di dalam patogenesis aterosklerosis. Namun, mekanisme molekul yang tepat yang mana sitokina mengawalatur pengekspresan gen PPARγ masih kurang difahami. Di dalam kajian ini, kami mengkaji kesan empat sitokina iaitu TNFα, IFNγ, IL-1α dan IL-1β ke atas pengekspresan mRNA, protein dan aktiviti pengikatan DNA PPARγ di dalam sel turunan makrofaj murin J774.2, model yang paling lazim digunakan untuk aterosklerosis. TNFα dan IFNγ didapati merencat pengekspresan mRNA dan protein PPARγ serta aktiviti pengikatan DNA. Sebaliknya, IL-1β merangsangkan peningkatan pengekspresan PPARγ pada peringkat mRNA, protein dan aktiviti pengikatan DNA. IL-1α pula tidak mempunyai kesan ke atas pengekspresan PPARγ dan aktiviti pengikatan DNA. Memandangkan perubahan dalam kandungan protein dan aktiviti pengikatan DNA di dalam makrofaj yang dirawat dengan sitokina selaras dengan perubahan dalam mRNA PPARγ, keputusan ini mencadangkan dengan kukuh bahawa pengekspresan PPARγ dan aktiviti pengikatan DNA dikawalatur pada peringkat metabolisme mRNA. Di antara empat sitokina yang digunakan, TNFα didapati paling berkesan di dalam merencat pengekspresan mRNA PPARγ. Ujian aktinomisin D menunjukkan bahawa paras ekspresi mRNA PPARγ dikawalatur pada peringkat kadar transkripsi gen, dan bukannya pada peringkat
kestabilan mRNA dalam sel J774.2 yang dirawat dengan TNFα. Penggunaan perencat-perencat spesifik terhadap laluan isyarat transduksi MAP kinas (PD98095, U0126, SB202190 dan SP600125) menunjukkan TNFα merencat paras mRNA PPARγ melalui laluan p42 ERK dan p46/54 JNK, yang kemudian mengaktifkan dan merangsang pengikatan c-Jun dan ATF2 ke elemen rangsangan cAMP (CRE) pada promoter mPPARγ1. Oleh itu, kajian ini menyediakan pandangan baru untuk laluan berpotensi yang mungkin terlibat di dalam pengawalaturan pengekspresan PPARγ oleh TNFα di dalam sel turunan makrofaj J774.2, dan mencadangkan satu sasaran berpotensi untuk halangan terapeutik terhadap aterosklerosis.
DIFFERENTIAL REGULATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPAR\(\gamma\)) BY CYTOKINES IN MURINE MACROPHAGE J774.2 CELL LINE: ELUCIDATION OF SIGNAL TRANSDUCTION PATHWAYS OF TUMOUR NECROSIS FACTOR ALPHA (TNF\(\alpha\)) IN REGULATING MACROPHAGE PPAR\(\gamma\) GENE EXPRESSION

ABSTRACT

Atherosclerosis is the leading cause of death in developed countries. The role of the PPAR\(\gamma\) in cytokine-activated macrophages is of crucial importance in the pathogenesis of atherosclerosis. However, the precise molecular mechanisms by which cytokines regulate PPAR\(\gamma\) gene expression are poorly understood. In the present study, we evaluated the effects of four cytokines i.e. TNF\(\alpha\), IFN\(\gamma\), IL-1\(\alpha\) and IL-1\(\beta\) on the expression of PPAR\(\gamma\) mRNA, protein and DNA binding activity in the murine macrophage J774.2 cell line, the widely used model for atherosclerosis. It was demonstrated that TNF\(\alpha\) and IFN\(\gamma\) inhibited the PPAR\(\gamma\) mRNA and protein expressions as well as DNA binding activity. By contrast, IL-1\(\beta\) induced a marginal increase at the levels of PPAR\(\gamma\) mRNA, protein content and DNA binding activity. IL-1\(\alpha\), however, had no significant effects on the PPAR\(\gamma\) gene expression and DNA binding activity. Since the changes observed in the PPAR\(\gamma\) protein content and DNA binding activity in cytokine-treated macrophages followed closely the corresponding changes in PPAR\(\gamma\) mRNA expression, the results strongly suggest that the PPAR\(\gamma\) expression and binding activity were mainly regulated at the levels of mRNA metabolism. Amongst four cytokines used, TNF\(\alpha\) was found to produce the most significant inhibition of PPAR\(\gamma\) mRNA expression. Actinomycin D experiment showed that the level of PPAR\(\gamma\) mRNA expression was mainly regulated at the level of rate of gene transcription and not at the level of mRNA
stability in TNFα-treated J774.2 cells. The use of specific inhibitors against MAP kinase signal transduction pathways (PD98095, U0126, SB202190 and SP600125) demonstrated that TNFα inhibited the mRNA levels of PPARγ via p42 ERK and p46/54 JNKs pathways, which in turn, activated and induced the binding of c-Jun and ATF2 to cAMP-responsive elements (CRE) in mPPARγ1 promoter. Thus, this study provides novel insights into the potential pathways that may be responsible for the molecular regulation of macrophage PPARγ gene expression by TNFα in macrophage J774.2 cell line, and suggests a potential target for therapeutic intervention against atherosclerosis.
CHAPTER 1

INTRODUCTION
1.1 Background

Atherosclerosis is the leading cause of death in the United States and the cause of more than half of all mortality in the developed countries. It is a long-term chronic disease characterized by the accumulation of lipids and fibrous connective tissue in the large arteries, accompanied by a local inflammatory response (Lusis, 2000). As the cholesterol plaque, or lesions, build up in the arteries over time, the risk for disease increases. Atherosclerotic coronary heart disease is the underlying cause for most heart attacks, and one of the most common causes for congestive heart failure, cardiac arrhythmias and sudden death (Lusis, 2000).

Epidemiological studies have revealed several genetic and environmental risk factors predisposing to atherosclerosis. Smoking, metabolic disorders clustering with insulin resistance, such as dyslipidemia, hypertension, diabetes, high cholesterol, and family history of heart disease, are particularly important risk factors. Predisposing symptoms of the disease include high blood pressure and elevated cholesterol, especially elevated LDL-cholesterol.

Research conducted during the past decade has led to an understanding of a relationship between the role of nuclear receptor peroxisome proliferator activated receptor $\gamma$ (PPAR$_\gamma$) in macrophage and the biological basis for arthrosclerosis (Tontonoz et al., 1998; Marx, 1998b; Chinetti, 1998; Ricote, 1999). For instance, PPAR$_\gamma$, upon activation, has been demonstrated to promote monocyte differentiation to macrophage and increase the uptake of oxidized LDL by macrophages to be transformed into foam cells (Tontonoz et al., 1998). It has also been shown to be highly
expressed in macrophage-derived foam cells and atherosclerotic plaque (Marx, 1998b). By contrast, PPAR\(\gamma\) has also been demonstrated to have an anti-atherogenic effect. For example, it was reported that PPAR\(\gamma\) is a potent negative regulator in the development of atherosclerosis (Ricote, 1999) and has the ability to induce apoptosis of human monocyte-derived macrophages (Chinetti, 1998).

1.2 Peroxisome proliferators activated receptors (PPARs)

Peroxisome proliferators activated receptors (PPARs) are a family of transcription factors that belong to the superfamily of nuclear receptors. The PPAR family consists of three distinct subtypes, termed \(\alpha\) (NR1C1), \(\beta/\delta\) (NR1C2) and \(\gamma\) (NR1C3), all of which display tissue-specific expression patterns reflecting their biological functions (Pineda-Torra et al., 2001).

All three PPAR isoforms possess similar structural and functional features. Principally, four functional domains have been identified, called A/B, C, D and E/F (Figure 1.1). The N-terminal A/B domain contains a ligand-independent activation function 1 (AF-1) (Werman et al., 1997) responsible for the phosphorylation of PPAR. The DNA binding domain (DBD) or C domain promotes the binding of PPAR to the peroxisome proliferator response element (PPRE) in the promoter region of target genes (Kliewer et al., 1992). The D site is a docking domain for cofactors. The E/F domain or ligand-binding domain (LBD) is responsible for ligand specificity and activation of PPAR binding to the PPRE, which increases the expression of targeted genes.
Figure 1.1 Schematic representation of the structural domains of PPAR. PPAR consists of four distinct functional domains. The A/B domain locates at the N-terminal with AF-1 is responsible for phosphorylation, the domain C is implicated in DNA binding, domain D is the docking region for cofactors and domain E/F is the ligand binding domain, containing AF-2, which promotes the recruitment of cofactors required for gene transcription.
Recruitment of PPAR co-factors to assist the gene transcription processes is carried out by the ligand-dependent activation function 2 (AF-2), which is located in the E/F domain (Berger & Moller, 2002).

Like other members of the nuclear receptor gene family, the PPAR subtypes possess a common domain structure which contains DNA-binding domains (DBD) and ligand-binding domains (LBD). Amino acid sequence comparison of DBD amongst PPAR subtypes shows they are highly conserved indicating that they share similar DNA binding site presence on the promoter region of the target genes, while the LBD have a slightly lower level of conservation across the subtypes (Figure 1.2) suggesting that they are ligand-specific. The NH$_2$-terminal domain of the subtypes shows low sequence identity which is responsible for differences in the biological function of the subtypes (Castillo et al., 1999).

### 1.3 Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$)

PPAR$\gamma$ was first identified as a component of an adipocyte differentiation-dependent regulatory factor (ARF6) that binds to the well-characterized, fat cell-specific enhancer of the adipocyte fatty acid-binding protein (aP2) gene (Tontonoz et al., 1994a; Tontonoz et al., 1994b).

PPAR$\gamma$, like the other PPARs, is an obligate heterodimer with another member of the nuclear receptor subfamily, the retinoic X receptors (RXR), the receptor for 9-cis-retinoic acid. Upon heterodimerization with RXR, PPAR$\gamma$ binds to peroxisome proliferator response element (PPRE) which in turn regulates downstream target genes (Figure 1.3) (Kliewer et al., 1992).
Figure 1.2 Comparison of amino acid identities of the DBD and LBD of human and mouse PPAR isoforms. Amino acid sequences are represented by open bars and numbers in the bars show the percentage of amino acid identity between human and mouse isoforms relative to PPARα. N, N-terminus; DBD, DNA-binding domain; LBD, ligand-binding domain and C, C-terminus (Murphy & Holder, 2000).
Figure 1.3 Gene transcription mechanisms of PPARγ. PPAR/RXR heterodimer binds to a PPRE in the regulatory regions of target genes, thereby governing the expression of the downstream target genes.
Structurally, PPRE consists of direct repeat of the nuclear receptor hexameric DNA core recognition motif AGGTCA separated by one nucleotide, known as DR-1 response elements (Lemberger et al., 1996; Juge-Aubry et al., 1997).

1.3.1 The structural organization of PPARγ gene

PPARγ has been cloned from a number of species, including mouse (Zhu et al., 1993; Kliewer et al., 1994), hamster (Aperlo et al., 1995), cattle (Sundvold et al., 1997), pig (Houseknecht et al., 1998) and human (Greene et al., 1995; Elbrecht et al., 1996).

The PPARγ gene, which has 9 exons (Figure 1.4) and extends over more than 100kb of genomic DNA for human (Fajas et al., 1997) and 105kb for mouse (Zhu et al., 1995), is mapped to chromosome 6 E3-F1 by in situ hybridization (Zhu et al., 1995).

In contrast to human, in which four PPARγ mRNA isoforms have been identified so far, i.e., PPARγ1, γ2 (Fajas et al., 1997), γ3 (Fajas et al., 1998) and γ4 (Sunvold & Lien, 2001), in mouse, only two PPARγ mRNA isoforms have been detected, termed PPARγ1 and γ2 (Zhu et al., 1995). The two mRNA isoforms of PPARγ arise as products of different promoter usage and alternative splicing from a single PPARγ gene, which differ only at their 5’ ends (Figure 1.4).
Figure 1.4 Structural organization of mPPAR\(\gamma\) gene. The eight exons (A1, A2, and 1-6) encoding the mPPAR\(\gamma1\) and the seven exons (B and 1-6) encoding the mPPAR\(\gamma2\) are shown in the genomic DNA. \(\gamma1P\) and \(\gamma2P\) represent the promoter of mPPAR\(\gamma1\) and mPPAR\(\gamma2\), respectively.
The PPAR\textsubscript{γ1} is encoded by 8 exons whereas PPAR\textsubscript{γ2} is encoded by 7 exons (Figure 1.4). Consistent with the production of two PPAR\textsubscript{γ} mRNAs, there are two PPAR\textsubscript{γ} promoters, each with a specific and distinctive expression pattern (Zhu et al., 1995). The two PPAR\textsubscript{γ} transcripts differ in their 5’end. PPAR\textsubscript{γ1} mRNA codes for one protein, while PPAR\textsubscript{γ2} codes for a different protein containing 28 additional amino acids at the N-terminus to the start codon of PPAR\textsubscript{γ1} for human (Sundvold et al., 1997) and 30 additional amino acids for mouse (Zhu et al., 1995).

In PPAR\textsubscript{γ1}, the two most upstream exons A1 and A2 comprise the 5’ untranslated region (UTR) and are spliced to the six most 3’ proximal exons (Kliewer et al., 1992) which encompass the common coding region shared by the two isoforms. The 5’ untranslated region (UTR) of PPAR\textsubscript{γ2} plus the additional 30 N-terminal amino acids specific to PPAR\textsubscript{γ2} are encoded by exon B, located between exon A2 and exon 1 (Zhu et al., 1995).

Thus, exons A1 and A2 are spliced with exon 1 to 6 to give rise to PPAR\textsubscript{γ1} mRNA. PPAR\textsubscript{γ2} mRNA is generated by splicing of exon B to exon 1 to 6. Each of the two zinc fingers of the DNA-binding domains of mPPAR\textsubscript{γ} is encoded by a separate exon (exon 2 and 3, respectively). The ligand-binding domain is encoded by two exons which are exons 5 and 6.
1.3.2 Tissue distribution and expression patterns of PPARγ

PPARγ mRNA is expressed in a tissue-specific manner. A comparison of the tissue-distribution of PPARγ transcripts among different species shows PPARγ mRNAs are specifically expressed at high levels in mammalian adipose tissue, large intestine and hematopoietic cells (Tontonoz et al., 1994b) and variable, but generally at lower levels in other tissues like kidney, liver and small intestine (Aperlo et al., 1995). Interestingly, PPARγ is barely detectable in muscle (Fajas et al., 1997; Auboeuf et al., 1997).

Analysis of the tissue distribution of the two PPARγ isoforms revealed that PPARγ1 shows rather ubiquitous distribution, whereas PPARγ2 had a more restricted distribution. PPARγ2 is much less abundant in all tissues analyzed compared to PPARγ1, the predominant PPARγ isoform. The only tissue expressing significant amounts of PPARγ2 is adipose tissue, where its mRNA makes up about 20% of total PPARγ mRNA (Fajas et al., 1997; Auboeuf et al., 1997).

Previous research showed that the expression of PPARγ2 mRNA is markedly increased very early during adipocyte differentiation (Chawla et al., 1994; Tontonoz et al., 1994b; Tontonoz et al., 1994c). Early induction of PPARγ2 expression during adipocyte differentiation and its adipose tissue selectivity suggesting its pivotal role in the regulation of adipocyte differentiation.
In addition to the role in adipocyte differentiation, PPAR\(\gamma\) has also been shown to play a pivotal role in monocytes differentiation. It was reported that PPAR\(\gamma\) is expressed in cells of the monocyte/macrophage lineage (Tontonoz et al., 1998; Greene et al., 1995; Ricote et al., 1998b; Jiang et al., 1998; Chinetti et al., 1998; Marx et al., 1998b) suggesting that PPAR\(\gamma\) is involved in the development of monocyte along the macrophage lineage, in particular in the conversion of monocytes to foam cell in the development of atherosclerosis (Tontonoz et al., 1998).

PPAR\(\gamma\) is also found expressed in several carcinomas, suggesting a role in the differentiation of cancer cell lines and in cell cycle regulation (Tontonoz et al., 1997; Altiok et al., 1997; Kubota et al., 1998; Mueller et al., 1998; DuBois et al., 1998).

1.3.3 Natural and synthetic ligands of PPAR\(\gamma\)

PPAR\(\gamma\) is a ligand-activated transcription factor. The binding of ligands to the receptor greatly increases its transcriptional activity. The ligand binding domain (LBD) of PPAR\(\gamma\) consist of 13 \(\alpha\) helices and a small four-stranded \(\beta\) sheet forming a large Y-shaped hydrophobic pocket (Figure 1.5). This pocket represents the ligand binding cavity and has a volume of approximately 1300 Å\(^3\), which is about twice that of the other nuclear receptors (Wagner et al., 1995).
Figure 1.5 Three-dimensional structure of ligand binding domains of PPARγ. An X-ray crystal structure of PPARγ (yellow ribbon) is shown. PPARγ is shown associated to LXXLL peptides (blue ribbons), the signature motif of the receptor coactivators. The solvent-accessible ligand binding pocket is displayed as an off-white surface (from Xu et al., 2001).
The PPAR\(\gamma\) ligands occupy \(~30\) –\(40\)% of the pocket, in contrast to the thyroid hormone receptor, where the ligand fills around \(90\)% of the pocket (Wagner et al., 1995). Besides its large size, another characteristic feature of the PPAR\(\gamma\) ligand binding pocket is that its bottom portion is sealed by helix 2', which is absent in other nuclear receptors. This particular helix may increase the size of the pocket, and possibly participates in an entry channel for the ligand.

The structural alignment of the ligand binding cavities of PPAR\(\gamma\) showed that the ligand selectivity depends on the identity of a single amino acid histidine in helix 5. This selectivity seems to be conserved between different ligand classes and corresponds to an intrinsic property of the receptors (Xu et al., 2001). The characteristics of the PPAR\(\gamma\) LBD give insight into the propensity of PPAR\(\gamma\) to interact with a variety of natural and synthetic compounds (Xu et al., 1999; Nolte et al., 1998).

A broad spectrum of synthetic and naturally occurring substances can serve as PPAR\(\gamma\) ligands, including pharmacological molecules, as well as fatty acids and fatty acid-derived products. PPAR\(\gamma\) is bound and activated by naturally occurring arachidonic acid metabolites derived from cyclooxygenase and lipoxygenase pathways, such as 15-deoxy-\(^{12,14}\)-prostaglandin J\(_2\) (15-dPGJ\(_2\)), 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) (Forman et al., 1995; Kliewer et al., 1995; Nagy et al., 1998; Huang et al., 1999) (Figure 1.6).
Figure 1.6 Natural ligands of PPARγ. PPARγ is activated by natural activators derived from fatty acids through the cyclooxygenase and lipoxygenase pathways such as 15-dPGJ₂, 12-HETE, 15-HETE, 9-HODE and 13-HODE.
In addition, other eicosanoids and unsaturated fatty acids are also reported to bind and activated PPARγ. This has been shown for the ω-3 polyunsaturated fatty acids, α-linolenic acid, eicosapentaenoic acid and docohexanoic acid (Krey et al., 1997; Kliewer et al., 1997). It was also shown that two eicosanoids present in oxidized low density lipoproteins (oxLDL) i.e. 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) are potent endogenous PPARγ ligands (Nagy et al., 1998) (Figures 1.6 and 1.7).

The synthetic compounds, thiazolidinediones (TZDs) or “glitazones” which include troglitazone, pioglitazone and rosiglitazone (Figure 1.8) are the first compounds reported as high-affinity PPARγ agonists (Lehmann et al., 1995). TZDs are currently being used for the treatment of insulin resistance and type II diabetes mellitus. TZD treatment results in a concomitant fall in glucose and insulin levels, through its insulin-enhancing action (Schwartz et al., 1998).

Non-TZDs such as isoxazolidinedione JTT-501 (Shibata et al., 1999) and the tyrosine-based GW-7845 (Figure 1.8) have PPARγ activation properties with significant anti-diabetic and anti-carcinogenic activities in rodents (Cobb et al., 1998; Suh et al., 1999).

Certain non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin and ibuprofen, had been shown to bind and activate PPARγ at high micromolar concentrations (Lehmann et al., 1997). Several other NSAIDs, including fenoprofen and flufenamic acid, were also shown to be weak PPARγ agonists (Lehmann et al., 1997).
Figure 1.7 Structure of natural ligands of PPARγ. 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15-dPGJ₂), eicosapentaenoic acid (EPA), 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) are potent PPARγ ligands.
Figure 1.8 Structure of synthetic agonists and antagonists of PPARγ. Troglitazone, pioglitazone, rosiglitazone, JTT-501, GW-7845 and CDDO are agonists of PPARγ; BADGE and LG-100641 are antagonists of PPARγ.
Novel PPAR\(\gamma\) partial agonists and antagonists have been recently identified. Triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid (CDDO) (Figure 1.8) is a partial agonist with anti-inflammatory properties (Wang et al., 2000). Bisphenol diglycidyl ether (BADGE) and LG-100641 (Figure 1.8) are recently identified PPAR\(\gamma\) antagonists (Wright et al., 2000; Mukherjee et al., 2000). Although these compounds have less clinical significance, they may be useful in understanding PPAR\(\gamma\) physiology and the identification of new ligands.

In addition to synthetic chemical methods, research in natural products has also yielded potent PPAR\(\gamma\) agonists from several medicinal plants. Saurufuran A from *Saururus chinensis* (Saururaceae) (Hwang et al., 2002), flavonoids such as chrysin, apigenin and kampferol (Liang et al., 2001) and phenolic compounds from *Glycyrrhiza uralensis* (Fabaceae) (Kuroda et al., 2003) are recently identified PPAR\(\gamma\) agonists.
1.3.4 Cofactors for the PPARγ

Cofactors have been shown to play an important part in the transcriptional control of PPARγ. They act as coactivators or corepressors that mediate the ability of PPARγ to initiate or suppress the transcription process. They interact with nuclear receptors in a ligand-dependent manner (Lemberger et al., 1996).

Initially, it was thought that the cofactors simply bridge PPARγ with the basic transcriptional machinery. However, it has become clear that these cofactors also carried several enzymatic activities, suggesting that they could control gene expression by specifically modifying chromatin and DNA structure (Glass et al., 1997; Pazin & Kadonaga, 1997; Moras & Gronemeyer, 1998). It is suggested that in the absence of any ligand, PPARγ may bind to corepressors which extinguish its transcriptional activity by the recruitment of histone deacetylases. Histone hypoacetylation is associated with condensed nucleosomes and thereby transcriptionally silent (Glass et al., 1997; Pazin & Kadonaga, 1997; Moras & Gronemeyer, 1998).

Ligand binding induces a conformational change in the receptor that results in the dissociation of corepressors and removal of histone deacetylases from DNA with subsequent recruitment of coactivator complexes that contain proteins with histone acetyltransferase activity. Acetylation is associated with changes of nucleosome conformation which modulates accessibility of promoter regions and facilitates transcription, thereby increases the transcription of target gene (Glass et al., 1997; Pazin & Kadonaga, 1997; Moras & Gronemeyer, 1998) (Figure 1.9).
Figure 1.9 Transcriptional activation of nuclear receptors. Transcriptional activation of nuclear receptors requires, in general, the release of corepressor (CoR) complexes, which contain histone deacetylase activity (HDAC), and the recruitment of coactivators (CoA), which target histone acetyl transferases (HAT) to the promoter. The differential docking of cofactors is facilitated by structural changes brought about by ligand-binding or receptor phosphorylation.
Some of these cofactors include members of two families of histone acetylases, i.e., CBP/p300 and steroid receptor coactivator (SRC)-1, as well as PPAR binding protein (PBP), PPAR gamma coactivator (PGC)-1 and silencing mediator for retinoid and thyroid hormone receptors (SMRT).

CBP and p300 were originally identified as CREB (cAMP-responsive binding protein) and E1 A interacting factors (Chrivia et al., 1993; Eckner et al., 1994; Janknecht & Hunter, 1996a; Janknecht & Hunter, 1996b). CBP/p300 are widely expressed (Misiti et al., 1998) and coactivate numerous transcription factors including several nuclear receptors (Chakravarti et al., 1996; Hanstein et al., 1996; Kamei et al., 1996; Smith et al., 1996; Dowell et al., 1997; Kraus & Kadonaga, 1998). CBP/p300 interacts with PPARγ through multiple domains in each protein (Gelman et al., 1999). Most notably, the NH2-terminal region of PPARγ can dimerize with CBP/p300 in the absence of ligand and this association enhances its constitutive AF-1 transcriptional activity (Gelman et al., 1999). The constitutive presence of CBP/p300 could enhance the basal ligand-independent transcriptional activity of PPARγ in vivo and could thereby explain the high level of basal activity of PPARγ.
1.4 Atherosclerosis

Atherosclerosis is a complex vascular disease initiated by accumulation and oxidation of plasma low-density lipoprotein (LDL) in the sub-endothelial space of the vessels. The development of atherosclerosis, however, is a complex long term process which involves recruitment and activation of different cell types, including monocytes/macrophages, endothelial cells, smooth muscle cells and T-lymphocytes in the intima of the arteries, thus leading to a local inflammatory response (Ross, 1999).

The trapped monocytes differentiate into macrophages that take up oxidized low-density lipoproteins (OxLDL) through scavenger receptors (SR), thus forming foam cells. Activated smooth muscle cells (SMC) proliferate and migrate from the media thus leading to neo-intima formation. Activation of these cells leads to the release of pro-inflammatory cytokines, which combined with the secretion of metalloproteases and expression of pro-coagulant factors, results in chronic inflammation and plaque instability. This can further evolve to plaque rupture and acute occlusion by thrombosis, resulting in myocardial infarction and stroke (Figure 1.10) [Ross, 1993; Ross, 1995; Ross, 1999; Lusis, 2000].

PPARγ has been reported to play an important role in the development of atherosclerosis. Interestingly, there are contradicting reports on the role of PPARγ in atherogenesis having demonstrated to produce pro-atherogenic effects in some contexts but anti-atherogenic effects in others.
Figure 1.10 The atherosclerosis process. (from Lusis, 2000).